Saccharomyces cerevisiae SSD1-V Confers Longevity by a Sir2p-Independent Mechanism

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ABSTRACT

The *SSD1* gene of *Saccharomyces cerevisiae* is a polymorphic locus that affects diverse cellular processes including cell integrity, cell cycle progression, and growth at high temperature. We show here that the *SSD1-V* allele is necessary for cells to achieve extremely long life span. Furthermore, addition of *SSD1-V* to cells can increase longevity independently of *SIR2*, although *SIR2* is necessary for *SSD1-V* cells to attain maximal life span. Past studies of yeast aging have been performed in short-lived *ssd1-d* strain backgrounds. We propose that *SSD1-V* defines a previously undescribed pathway affecting cellular longevity and suggest that future studies on longevity-promoting genes should be carried out in long-lived *SSD1-V* strains.

AGING in *Saccharomyces cerevisiae* can be studied by mutations that extend the replicative life span of mother cells, defined as the number of daughters produced by a given mother cell prior to senescence. One cause of aging in yeast is the accumulation of extrachromosomal ribosomal DNA circles (ERCs), circular DNA molecules derived from homologous recombination within the ribosomal DNA (rDNA; Sinclair and Guarente 1997). ERCs are self-replicating and asymmetrically segregated to the mother-cell nucleus during S-phase, resulting in an exponential increase in ERC copy number with age and, ultimately, in cell death (Sinclair and Guarente 1997).

One important determinant of yeast longevity is the Sir2 protein (Kaeberlein *et al.* 1999). Sir2p is an NAD-dependent histone deacetylase (Imai *et al.* 2000; Landry *et al.* 2000; Smith *et al.* 2000) required for transcriptional silencing at telomeres (Gottschling *et al.* 1990), silent mating (*HM*) loci (Ivy *et al.* 1986; Rine and Herskowitz 1987), and the rDNA (Bryk *et al.* 1997; Smith and Boeke 1997). Mutation of *SIR2* results in increased rDNA recombination (Gottlieb and Esposito 1989), increased ERC formation (Kaeberlein *et al.* 1999), and decreased life span (Kennedy *et al.* 1995), whereas overexpression extends life span by 30–40% (Kaeberlein *et al.* 1999). Sir2p is also required for life-span extension by calorie restriction (CR), demonstrating the importance of this protein as a central regulator of longevity (Lin *et al.* 2000). Overexpression of a Sir2p homolog, Sir-2.1, has been shown to extend life span in the nematode *Caenorhabditis elegans*, suggesting that Sir2 proteins regulate aging in higher eukaryotes as well (Tissenbaum and Guarente 2001).

SSD1 is a polymorphic locus that affects diverse cellular processes. Two allele classes, designated *SSD1-V* and *ssd1-d*, have been identified for *SSD1*. *SSD1-V* alleles confer viability in the absence of the Sit4 protein phosphatase and code for functional Ssd1 protein. In contrast, strains carrying *ssd1-d* alleles are inviable in the absence of Sit4p (Sutton *et al.* 1991), and d-type alleles are likely null for Ssd1p function. Both V- and d-type alleles have been found in natural isolates and in laboratory strains of *S. cerevisiae*. A recent report (Wheeler *et al.* 2003) suggests that the *SSD1* allele type affects pathogenicity of yeasts, indicating that allelic variation at the *SSD1* locus may be important for survival under various environmental conditions.

A potential role for *SSD1-V* as a regulator of cell life span was suggested by the observation that *SSD1-V* suppresses many phenotypes associated with mutation of the *MPT5/UTH4* gene (Kaeberlein and Guarente 2002). *MPT5* is a post-transcriptional regulator (Tadauchi *et al.* 2001) involved in regulating the pheromone response (Chen and Kurjan 1997), cell-wall stability (Kaeberlein and Guarente 2002), telomere silencing (Cockell *et al.* 1998), and longevity (Kennedy *et al.* 1995). Like *SIR2*, *MPT5* is a limiting factor for longevity: overexpression of *MPT5* extends life span, whereas the deletion of *MPT5* has the opposite effect (Kennedy *et al.* 1997).

SSD1-V suppresses the temperature-sensitive growth defect caused by mutation of *MPT5* as well as the sensitivity to calcofluor white (CFW) and sodium dodecyl sulfate (SDS; Kaeberlein and Guarente 2002). In strains lacking *SSD1-V*, deletion of *MPT5* is synthetically lethal

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TABLE 1

Yeast strains used in this study

Strain	Relevant genotype
PSY316AR	*MAT*α *ADE2::rDN1 ade2-101 his3-*Δ*200 leu2-3,112 lys2-801 ura3-52 ssd1-d*
MKY2018	PSY316AR pRS406/*URA3*
MKY2012	PSY316AR *SSD1-V/URA3*
MK2017	PSY316AR *SSD1-V/LEU2*
MKY2076	PSY316AR *ssd1-d*Δ*::HIS3*
MKY2077	PSY316AR *ssd1-d*Δ*::HIS3 SSD1-V/LEU2*
MKY2013	PSY316AR *sir2::kanMX*
MKY2014	PSY316AR *sir2::kanMX SSD1-V/URA3*
MKY2085	PSY316AR *sir2*Δ*::kanMX fob1*Δ*::LEU2* pRS406/*URA3*
MKY2086	PSY316AR *sir2*Δ*::kanMX fob1*Δ*::LEU2 SSD1-V/URA3*
MKY2147	PSY316AR *cyt1*Δ*::kanMX*
MKY2153	PSY316AR *cyt1*Δ*::kanMX SSD1-V/URA3*
MKY2020	PSY316AR *mpt5*Δ*::LEU2*
MKY2021	PSY316AR *mpt5*Δ*::LEU2 SSD1-V/URA3*
MKY2152	PSY316 *pADH_NCA3*
MKY2149	PSY316AR *nca3::HIS3*
MKY2152	PSY316AR *nca3::HIS3 SSD1-V/URA3*
MKD202	PSY316AR a/α *sit4::HIS3/SIT4 SSD1-V/URA3*
BKY5	*MAT***a** *ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 uth4-14c ssd1-d*
MKY3021	BKY5 *SSD1-V/URA3*
W303R	*MAT***a** *ADE2::rDN1 his3 leu2 trp1 ura3 ssd1-d2 RAD5*
MKY1324	W303R *mpt5::LEU2*
MKY1332	W303R *mpt5::LEU2 SSD1-V/URA3*
MKY183	W303R *sir2::TRP1*
MKY596	W303R *pADH_MPT5*
MKY606	W303R *sir2::TRP1 pADH_MPT5*
MKY580	W303R *rpd3::LEU2*
MKY574	W303R *sir2::TRP1 rpd3::URA3*
MKY590	W303R *mpt5::LEU2 rpd3::URA3*

in combination with loss of function in either of the SBF or *CCR4* transcriptional complexes (Kaeberlein and Guarente 2002), both of which function downstream of protein kinase C (Pkc1p) to promote cell-wall biosynthesis (Igual *et al.* 1996; Madden *et al.* 1997; Chang *et al.* 1999). These results were interpreted to suggest that Mpt5p, Ssd1p, and Pkc1p define three parallel pathways that function to ensure cell integrity (Kaeberlein and Guarente 2002).

In addition to suppressing the cell integrity defects, *SSD1-V* suppresses the shortened life span caused by deletion of *MPT5* (Kaeberlein and Guarente 2002). These observations raise the possibility that *SSD1-V* might also promote longevity in wild-type cells. Here we show that addition of a single copy of *SSD1-V* to *ssd1-d* wild-type cells extends life span in at least two different strain backgrounds. Furthermore, life-span extension by *SSD1-V* does not require the Sir2 protein, although the presence of both *SSD1-V* and *SIR2* is necessary for maximal longevity.

MATERIALS AND METHODS

Strains and genetic techniques: The strains used in this study are listed in Table 1. All strains were derived from W303R (described in Mills *et al.* 1999), PSY316 (described in Park *et al.* 1999), or BKY5 (described in Kennedy *et al.* 1995). Genetic crosses, sporulation, and tetrad analysis were carried out as described (Sherman and Hicks 1991). The genotype of inviable spore clones was inferred, when possible, on the basis of marker segregation in viable spore clones from the same tetrad. Unless otherwise noted, cells were cultured in YPD or synthetic media prepared using conventional methods (Guthrie and Fink 1991). Yeast transformation was accomplished by the lithium acetate method (Gietz *et al.* 1992). All other gene deletions were generated by transforming cells with PCR-amplified disruption cassettes as described (Kaeberlein *et al.* 1999). In each case, the entire open reading frame was removed. All disruptions were verified phenotypically or by PCR. The *SSD1-V* integrating plasmids p406SSD1 and p405SSD1 were previously described (Kaeberlein and Guarente 2002). Unless otherwise indicated, all *SSD1-V* strains contain *SSD1-V* integrated at the marker locus and still carry the *ssd1-d* allele at the *SSD1* locus. Deletion of *ssd1-d* does not affect any of the phenotypes tested, including life span, growth at 30°, 37°, or 40°, or sensitivity to calcofluor white.

Determination of *SSD1* allele: To determine which *SSD1* allele was present in PSY316, one copy of the *SIT4* gene was deleted in diploid cells. Sporulation of these cells revealed that deletion of *SIT4* always resulted in lethality in haploid spore clones ($n > 20$ *sit4*Δ spores). This lethality was suppressed by integration of a single copy of *SSD1-V* at the *URA3* locus. Therefore, we conclude that in PSY316 the *SSD1* allele type is *ssd1-d*.

Life span, recombination, and ERC analysis: Life spans were performed as described (Kaeberlein and Guarente 2002). Statistical significance was determined by a Wilcoxon rank-sum test. Average life span is different for $P < 0.05$. Figures 1–5 represent data derived from a single experiment, unless otherwise stated. ERC levels were determined as described (Defossez *et al.* 1999; Kaeberlein *et al.* 1999). ERCs were separated on a 0.6% agarose gel without addition of ethidium bromide at 1 V/cm for 48 hr. rDNA recombination rate was determined as described (Kaeberlein *et al.* 1999).

Microarray analysis: RNA isolation and microarray analysis were performed essentially as described (Lin *et al.* 2002). For the purposes of comparative statistical analyses, candidate genes with altered transcript levels in *SSD1-V* cells relative to wild type were defined as such if the average ratio of *SSD1-V* (Cy5) to *ssd1-d* (Cy3) was >1.5 or <0.667 (1.5-fold decrease) in three independent experiments. All such genes are listed in supplemental Table 1 at http://www.genetics.org/supplemental/). As a control, two independent microarray analyses were performed on *ssd1*Δ (Cy5) cells relative to *ssd1-d* (Cy3) cells. The 124 genes defined as regulated by CR represent the subset of genes found to show significant changes in mRNA expression both in cells lacking *HXK2* and in cells grown on 0.5% glucose (Lin *et al.* 2002). Statistical significance of the overlap between regulated genes in different experiments shown in Table 2 was calculated using a hypergeometric distribution. *P* values were obtained from the online hypergeometric distribution calculator at http://www.alewand.de/stattab/tabdiske.htm. Gene function annotation was obtained from

TABLE 2

Overlap in gene expression profiles with CR

Life-span-extending mutation/intervention	No. of genes differentially transcribed	Overlap with CR	*P* value
SSD1-V	97	6	0.01
HAP4 overexpression	255	55	$<10^{-30}$
High osmolarity	117	48	$<10^{-30}$
Calorie restriction	124	124	—

Microarray analysis was performed on logarithmically growing cells in YPD for each of the long-lived cell types listed. The *P* value was calculated using a hypergeometric distribution and represents the probability that the number of genes similarly regulated by two life-span-extending interventions would be observed by chance. All microarray data sets are available at http://web.mit.edu/biology/guarente/arrays/kaeberlein (see MATERIALS AND METHODS).

the Saccharomyces Genome Database. Supplemental Table 1, normalized ratio (Cy5/Cy3), and spot intensity data sets for all experiments presented in this article are available at http://web.mit.edu/biology/guarente/arrays/kaeberlein.

RESULTS

***SSD1-V* extends life span and improves growth at high temperature:** Mpt5p is a limiting factor for longevity and functions in a pathway parallel to *SSD1-V* for cell integrity (KAEBERLEIN and GUARENTE 2002). On the basis of this genetic interaction, we hypothesized that *SSD1-V* might also regulate longevity. We first determined that our wild-type strain PSY316 carries the *ssd1-d* allele at the *SSD1* locus, on the basis of the inviability caused by deletion of *SIT4* in this background (see MATERIALS AND METHODS). A single copy of *SSD1-V* integrated at the *URA3* locus results in an ~50% increase in mean life span (Figure 1A). Integration of *SSD1-V* at the *SSD1* locus has a similar effect on life span (not shown). Deletion of the chromosomal *ssd1-d* allele of PSY316 has no effect on life span and does not affect life-span extension by *SSD1-V* (Figure 1A). Therefore, *ssd1-d* is a null allele with respect to life span. All subsequent experiments were carried out in the parental *ssd1-d* background.

PSY316 is a moderately long-lived yeast strain; however, many yeast aging studies have been carried out in short-lived strain backgrounds having mean life spans of 10–15 generations. To determine whether the life-span extension by *SSD1-V* was strain specific, we integrated *SSD1-V* into the short-lived strain BKY5. We verified that BKY5 carries an *ssd1-d* allele (see MATERIALS AND METHODS) as well as a previously identified C-terminal truncated allele of *MPT5* (KENNEDY *et al.* 1997). Addition of *SSD1-V* to BKY5 results in an 85% increase in mean life span (Figure 1B). Thus, *SSD1-V* promotes long life span in at least two different *ssd1-d* strain backgrounds.

We had previously observed that cells from strain PSY316 grow normally at 37°, but are incapable of sustained growth at 40°. Cells grown at 40° generally arrest as large-budded cells with a significant fraction undergoing lysis (data not shown), consistent with a loss of cell-wall integrity at the restrictive temperature. Addition of *SSD1-V* fully suppresses these phenotypes and allows growth of PSY316 at 40° (Figure 1C). Addition of *SSD1-V* to PSY316 also improves growth in the presence of the cell-wall-perturbing agents CFW and SDS (data not shown), as previously reported for strain W303R (KAEBERLEIN and GUARENTE 2002).

***SSD1-V* extends life span in the absence of *SIR2*:** The Sir2 protein is a central regulator of yeast longevity, necessary for life-span extension in response to environmental signals such as reduced nutrient availability (LIN *et al.* 2000) and osmotic stress (KAEBERLEIN *et al.* 2002). To place *SSD1-V* into a genetic pathway relative to Sir2p, we integrated the *SSD1-V* allele into a strain lacking *SIR2*. Deletion of *SIR2* shortens wild-type life span by ~50% (KAEBERLEIN *et al.* 1999). Surprisingly, addition of *SSD1-V* resulted in a significant life-span extension in the absence of Sir2p (Figure 2A), although *sir2 SSD1-V* cells are shorter lived than *SIR2* wild-type cells.

Due to the extremely short life span caused by lack of Sir2p, we also wished to determine the effect of *SSD1-V* on *sir2 fob1* double-mutant cells, which have an almost wild-type life span (KAEBERLEIN *et al.* 1999). As predicted by our previously proposed model (LIN *et al.* 2000, 2002), CR by growth on low glucose fails to extend life span in the absence of *SIR2* (Figure 2B). In contrast, *sir2 fob1 SSD1-V* cells grown on 2% glucose have a life span that is significantly longer than that of *sir2 fob1 ssd1-d* cells (Figure 2C). However, as was the case for *sir2 SSD1-V* cells, *sir2 fob1 SSD1-V* cells do not live as long as *SIR2 FOB1 SSD1-V* cells. Therefore, *SSD1-V* acts in a novel, *SIR2*-independent pathway for longevity, although Sir2p is required for maximum longevity in *SSD1-V* cells.

The Sir2-dependent life-span extension caused by CR is the result of a metabolic shift from fermentation to respiration (LIN *et al.* 2002). It is possible that a portion

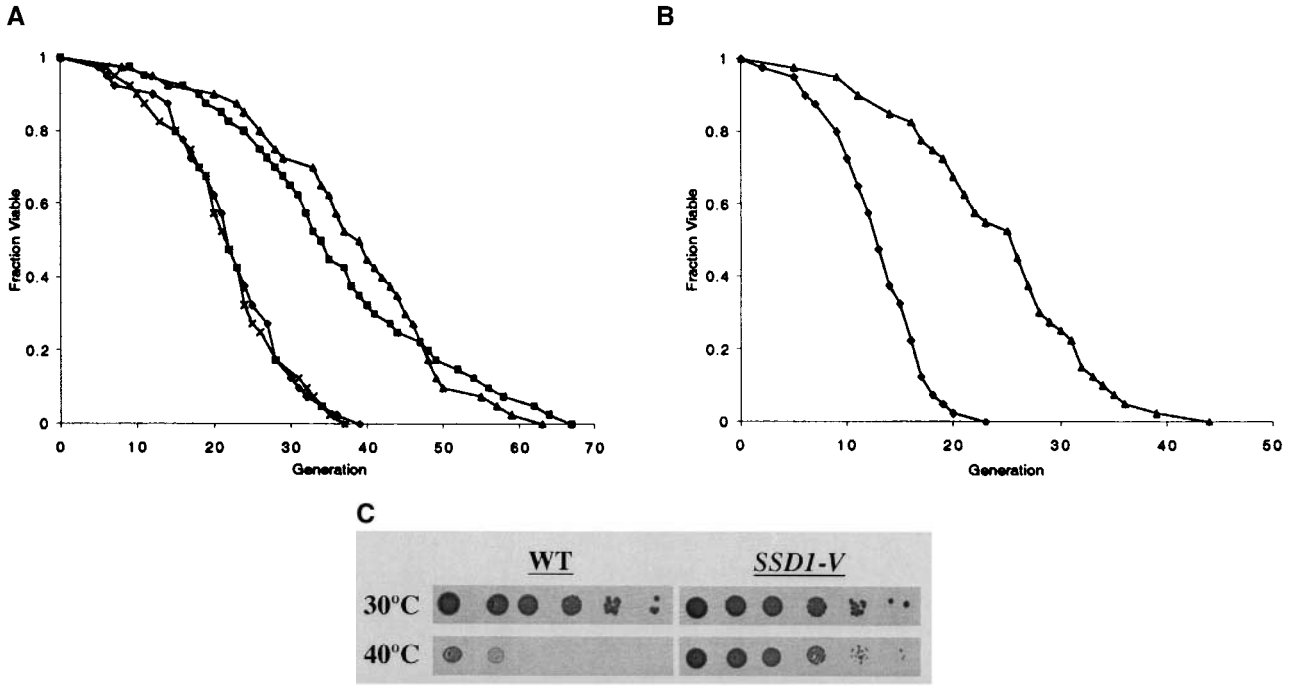

FIGURE 1.—*SSD1-V* extends life span and improves cell integrity. (A) Life spans were determined for PSY316 (◆), PSY316 *SSD1-V* (■), PSY316 *ssd1*Δ*::HIS3* (X), and PSY316 *ssd1*Δ*::HIS3 SSD1-V* (▲). Mean life spans and number of cells analyzed were PSY316 22.2 ($n = 40$), PSY316 *SSD1-V* 36.2 ($n = 40$), PSY316 *ssd1*Δ*::HIS3* 21.9 ($n = 40$), and PSY316 *ssd1*Δ*::HIS3 SSD1-V* 38.0 ($n = 40$). (B) Life spans were determined for BKY5 (◆) and BKY5 *SSD1-V* (▲). Mean life spans and number of cells analyzed were BKY5 13.0 ($n = 40$) and BKY5 *SSD1-V* 24.3 ($n = 40$). (C) PSY316 cells are unable to grow at temperatures >39° unless *SSD1-V* is present. Tenfold serial dilutions of a log-phase culture plated onto YPD and incubated at either 30° or 40° for 48 hr are shown.

of the longevity conferred by *SSD1-V* is achieved by causing the cell to undergo a similar metabolic shift. To address this possibility, the effect of *SSD1-V* on life span was examined in a respiration-deficient strain lacking the *CYT1* gene encoding cytochrome c1. Mutation of *CYT1* prevents life-span extension by CR or by overexpression of the Hap4p transcription factor (LIN *et al.* 2002). In contrast, *SSD1-V* extends the life span of cells lacking *CYT1* to the same extent as that of wild-type cells (Figure 2D), suggesting that the mechanism of life-span extension by *SSD1-V* is independent of mitochondrial function and respiration.

High osmolarity extends the life span of *ssd1-d* but not of *SSD1-V* cells: We have previously demonstrated that the osmotic concentration of media is a determining factor for mother-cell life span. Addition of 1 M sorbitol suppresses the short life span and cell-wall defects of *mpt5 ssd1-d* cells in the W303 strain background (KAEBERLEIN and GUARENTE 2002). Growth on YPD supplemented with 1 M sorbitol (YPDS), 1 M xylitol, or 1 M glucose also extends the life span of *MPT5 ssd1-d* cells in PSY316 through a *SIR2*-dependent mechanism (KAEBERLEIN *et al.* 2002). We were therefore interested in determining whether high osmolarity would affect the life span of long-lived *SSD1-V* cells.

As observed in W303R, growth on YPDS suppressed the temperature sensitivity and short life span of *mpt5 ssd1-d* cells in the PSY316 strain background (not shown). Growth on YPDS also dramatically increased life span in wild-type *ssd1-d* cells by ~60% (Figure 2E). In contrast, growth on YPDS resulted in only a modest 10% increase in the life span of *SSD1-V* cells.

***SSD1-V* acts independently of ERC formation or accumulation:** Sir2p and calorie restriction promote longevity by decreasing the formation and accumulation of ERCs in mother cells (KAEBERLEIN *et al.* 1999; LIN *et al.* 2000). To determine whether the long life span of *SSD1-V* cells is also due to fewer ERCs, we measured the rate of ERC formation and the amount of ERCs present in *ssd1-d* and *SSD1-V* cells. ERC formation was estimated by determining the frequency at which an *ADE2* marker integrated into the rDNA is lost, as demonstrated by the presence of half-sectored colonies (KAEBERLEIN *et al.* 1999). No difference was observed between *SSD1-V* and *ssd1-d* cells by this assay (Figure 3A). Upon direct quantitation of ERCs from unsorted cells, we observed that *SSD1-V* cells often had higher levels of ERCs than *ssd1-d* cells (Figure 3B), indicating that the life-span extension caused by *SSD1-V* is unlikely to be the result of decreased ERC formation or accumulation. This is consistent with the observation that *SSD1-V* does not require Sir2p to extend life span and may indicate that *SSD1-V* increases the resistance of cells to ERCs (see DISCUSSION).

Transcriptional analysis of *SSD1-V*: We previously ob-

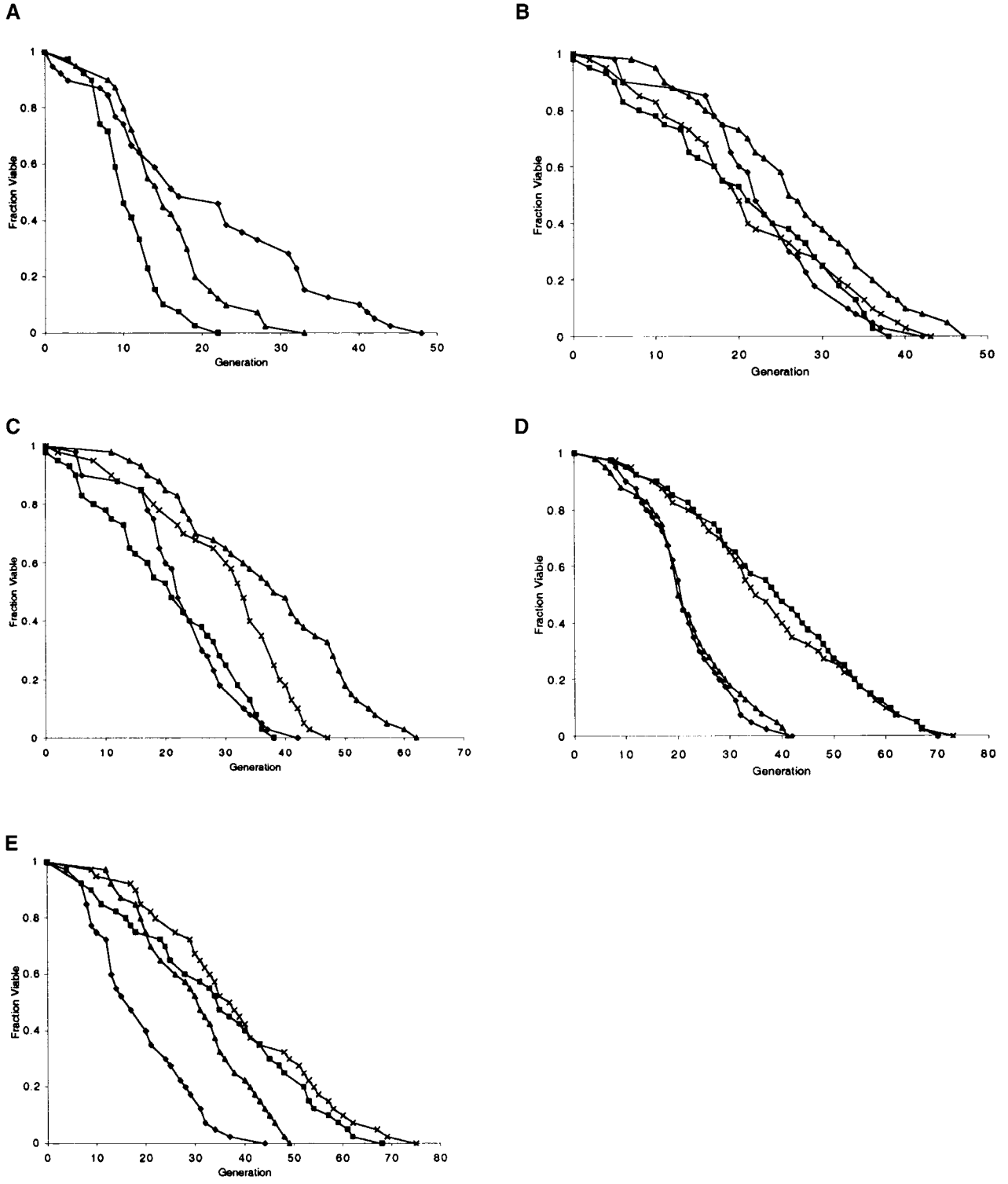

FIGURE 2.—*SSD1-V* and Sir2p act in different pathways to promote longevity. (A) Life spans were determined for PSY316 (◆), PSY316 *sir2* (■), and PSY316 *sir2 SSD1-V* (▲). Mean life spans and number of cells analyzed were PSY316 21.2 (n = 40), PSY316 *sir2* 10.8 (n = 39), and PSY316 *sir2 SSD1-V* 15.7 (n = 40). (B) Life spans were determined for PSY316 (◆), PSY316 *sir2 fob1* (■), PSY316 0.5% glucose (▲), and PSY316 *sir2 fob1* 0.5% glucose (X). Mean life spans and number of cells analyzed were PSY316 22.8 (n = 40), PSY316 *sir2 fob1* 21.2 (n = 39), PSY316 0.5% glucose 27.0 (n = 40), and PSY316 *sir2 fob1* 0.5% glucose 21.4 (n = 40). (C) Life spans were determined for PSY316 (◆), PSY316 *sir2 fob1* (■), PSY316 *SSD1-V* (▲), and PSY316 *sir2 fob1 SSD1-V* (X). Mean life spans and number of cells analyzed were PSY316 22.8 (n = 40), PSY316 *sir2 fob1* 21.2 (n = 39), PSY316 *SSD1-V* 37.3 (n = 40), and PSY316 *sir2 fob1 SSD1-V* 30.0 (n = 40). (D) Life spans were determined for PSY316 (◆), PSY316 *SSD1-V* (■), PSY316 cyt1 (▲), and PSY316 *SSD1-V* cyt1 (X). Mean life spans and number of cells analyzed were PSY316 21.7 (n = 40), PSY316 *SSD1-V* 39.3 (n = 40), PSY316 cyt1 22.2 (n = 40), and PSY316 *SSD1-V* cyt1 37.9 (n = 40). (E) Life spans were determined for PSY316 YPD (◆), PSY316 *SSD1-V* YPD (■), PSY316 YPDS (▲), and PSY316 *SSD1-V* YPDS (X). Mean life spans and number of cells analyzed were PSY316 YPD 19.1 (n = 40), PSY316 *SSD1-V* YPD 34.8 (n = 40), PSY316 YPDS 30.4 (n = 40), and PSY316 *SSD1-V* YPDS 39.1 (n = 40).

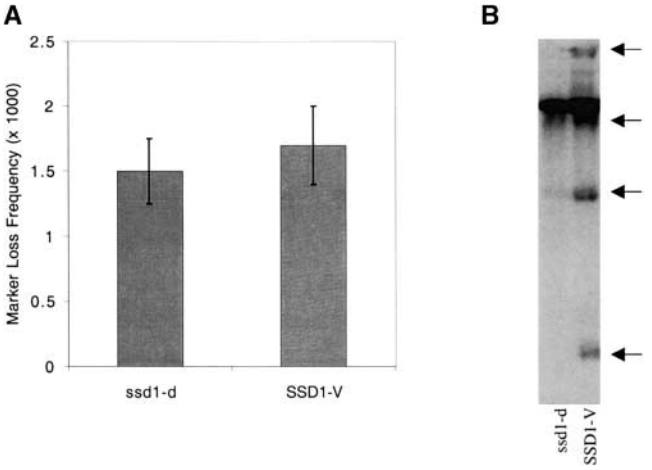

FIGURE 3.—*SSD1-V* does not extend life span by decreasing ERC levels. (A) *SSD1-V* has no detectable effect on rDNA recombination. rDNA recombination was measured by the frequency at which an *ADE2* marker integrated into the rDNA is lost. A total of 33,000 colonies from three independently derived isolates were examined for each strain. (B) DNA from unsorted cells was isolated and electrophoresed as described (KAEBERLEIN *et al.* 1999). The gel was transferred and probed with sequence homologous to the rDNA. ERCs are denoted by arrows. The dark band present in both lanes corresponds to genomic rDNA. In this isolate, the long-lived *SSD1-V* cells have a greater steady-state amount of ERCs than do *ssd1-d* cells, as quantitated by the ratio of ERC DNA to genomic rDNA. However, in one other independently derived *SSD1-V* transformant we were unable to detect a significant difference in ERC levels relative to those in wild-type cells. In no case did we detect fewer ERCs in *SSD1-V* cells.

served that calorie restriction by growth in 0.5% glucose, which promotes long life span, causes characteristic changes in gene expression that are reproduced in two genetic models of CR, namely overexpression of *HAP4* and deletion of *HXK2* (LIN *et al.* 2002). More recently, we demonstrated that growth in the presence of high external osmolarity also extends life span in a *SIR2*-dependent manner and results in a gene expression profile with significant similarity to calorically restricted cells (KAEBERLEIN *et al.* 2002). Although *SSD1* has been implicated in many different genetic pathways, little is known regarding the function of the Ssd1 protein. To further understand the effect of *SSD1-V* on cell physiology and longevity, we used microarray analysis to examine the transcriptional profile of *SSD1-V* cells relative to wild-type *ssd1-d* cells.

Messenger RNA was harvested from logarithmically growing *ssd1*Δ, *ssd1-d*, or *SSD1-V* cells. Using RNA derived from three independent experiments, a total of 97 genes were observed to undergo a change in expression >1.5-fold in *SSD1-V* cells relative to *ssd1-d* cells (supplemental Table 1 at http://www.genetics.org/supplemental/). Of these 97 genes, only 6 underwent similar transcriptional changes in calorically restricted cells (Table 2). This is only slightly greater than the number of genes expected to overlap between the *SSD1-V* and CR data sets by chance and is in contrast to the highly significant overlap in transcriptional changes observed between CR and *HAP4* overexpression (LIN *et al.* 2002) or between CR and high external osmolarity (KAEBERLEIN *et al.* 2002). Intriguingly, of the 6 genes that show similar transcriptional changes in calorically restricted cells and *SSD1-V* cells, 4 are involved in iron-siderochrome transport: *FIT1*, *FIT2*, *FIT3*, and *ARN1* (supplemental Table 1 at http://www.genetics.org/supplemental/).

***NCA3* mRNA is increased in *SSD1-V* cells:** One particularly interesting candidate gene that shows altered mRNA levels in *SSD1-V* cells is *NCA3* (supplemental Table 1 at http://www.genetics.org/supplemental/). Nca3p is a member of the SUN family of proteins (Sim1, Uth1, Nca3, and Sun4) and functions to promote maturation of the mitochondrially encoded *ATP8-ATP6* cotranscript (PELISSIER *et al.* 1995). Upregulation (3.7-fold) of *NCA3* by *SSD1-V* is striking because Nca3p shares extensive homology (60%) with the aging protein Uth1p. Interestingly, we find that *NCA3* mRNA is increased 4-fold in long-lived cells lacking Uth1p (data not shown). Overexpression of *NCA3* from the *ADH1* promoter results in a slight, but reproducible, increase in life span (Figure 4A), suggesting that Nca3p dosage can affect longevity. However, *NCA3* is not required for the majority of the life-span extension seen in *SSD1-V* cells, as demonstrated by the finding that *nca3 SSD1-V* cells have a life span comparable to that of *NCA3 SSD1-V* cells (Figure 4B). Therefore, we conclude that increased transcription of *NCA3* accounts for, at most, a minor fraction of the longevity-promoting activity of *SSD1-V*.

***MPT5* and *SIR2* affect longevity in a pathway parallel to *SSD1-V*:** We have previously demonstrated that *MPT5* and *SSD1-V* act in parallel pathways to promote cell integrity and that *SSD1-V* suppresses the short life span caused by deletion of *MPT5* in the W303R strain background (KAEBERLEIN and GUARENTE 2002). As expected, addition of *SSD1-V* similarly suppresses the short life span of cells lacking *MPT5* in PSY316 (Figure 5A). It is interesting to note, however, that *mpt5 SSD1-V* cells have a life span intermediate between cells lacking both *MPT5* and *SSD1-V* and cells with functional copies of both genes. In fact, *mpt5 SSD1-V* cells have a life span not significantly different from that of the wild-type *MPT5 ssd1-d* strain, suggesting that *MPT5* and *SSD1-V* have additive effects on longevity, as would be expected for genes functioning in parallel pathways.

Like *SSD1-V*, overexpression of *MPT5* increases mother-cell life span (KENNEDY *et al.* 1997). Since *SSD1-V* is capable of extending the life span of cells lacking *SIR2*, we wished to determine whether overexpression of *MPT5* would have a similar effect. In contrast to *SSD1-V*, *MPT5* overexpression fails to extend the life span of *sir2 fob1* cells (Figure 5B), suggesting that *MPT5* and *SIR2* act in the same pathway to promote longevity. Furthermore, overexpression of *SIR2* fails to further extend the life span of cells in which *MPT5* is overexpressed (Figure 5B).

It was previously observed that cells with altered dos-

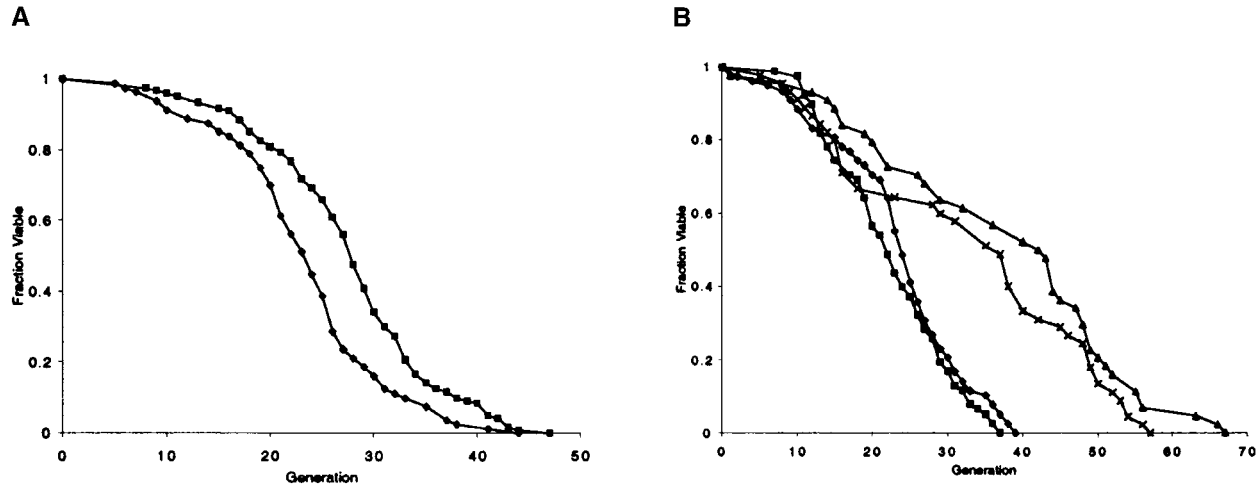

FIGURE 4.—Effect of *NCA3* on cell life span. (A) Life spans were determined for PSY316 (◆) and PSY316 *pADH_NCA3* (■). Mean life spans and number of cells analyzed were PSY316 23.4 (n = 80) and PSY316 *pADH_NCA3* 27.6 (n = 120). Data were pooled from two different experiments. (B) Life spans were determined for PSY316 (◆), PSY316 *nca3* (■), PSY316 *SSD1-V* (▲), and PSY316 *nca3 SSD1-V* (X). Mean life spans and number of cells analyzed were PSY316 23.3 (n = 40), PSY316 *nca3* 22.3 (n = 40), PSY316 *SSD1-V* 37.5 (n = 40), and PSY316 *nca3 SSD1-V* 32.8 (n = 40).

age of *MPT5* display changes in telomeric and rDNA silencing (KENNEDY 1996), suggesting a further link between *MPT5* and *SIR2*. Overexpression of *MPT5* increases rDNA silencing and decreases telomeric silencing, while deletion has an opposite effect (Table 3). Integration of *SSD1-V*, in contrast, has no detectable effect on silencing at either locus. Consistent with the inability of *MPT5* overexpression to extend life span in the absence of *SIR2*, the enhanced rDNA silencing observed in cells overexpressing *MPT5* is fully suppressed by deletion of *SIR2* (Table 3). On the basis of these results, we propose that overexpression of *MPT5* increases life span by relocalizing Sir2p from telomeres to the rDNA (see DISCUSSION), thus enhancing the ability of Sir2p to inhibit ERC accumulation in aging mother cells.

DISCUSSION

One cause of aging in yeast is the accumulation of ERCs (SINCLAIR and GUARENTE 1997). A central regulator of ERC formation and longevity is the Sir2p histone deacetylase (KAEBERLEIN *et al.* 1999). Several genes that

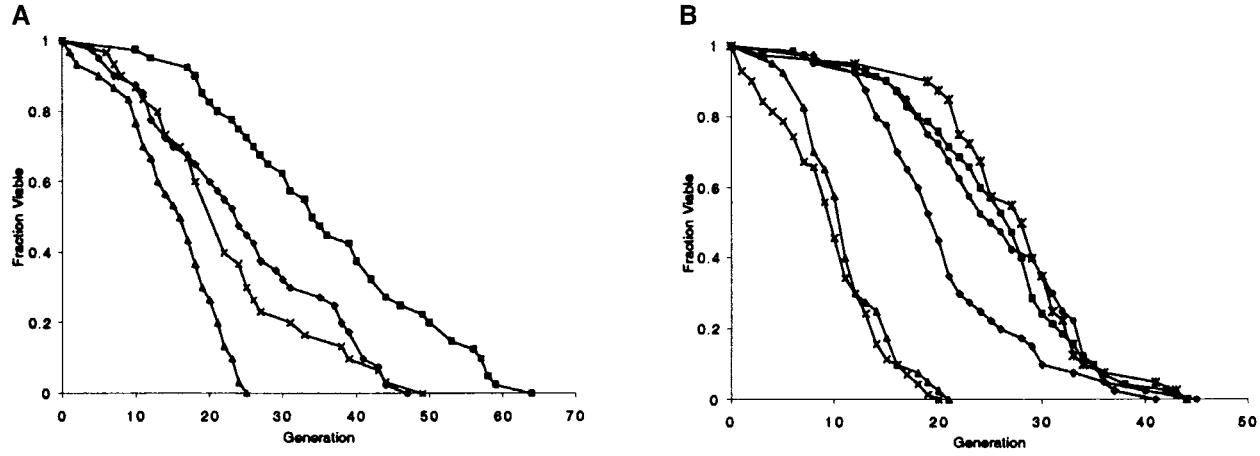

FIGURE 5.—*MPT5* and *SIR2* determine longevity in a pathway parallel to *SSD1-V*. (A) Life spans were determined for PSY316 (◆), PSY316 *SSD1-V* (■), PSY316 *mpt5* (▲), and PSY316 *mpt5 SSD1-V* (X). Mean life spans and number of cells analyzed were PSY316 24.3 (n = 40), PSY316 *SSD1-V* 35.5 (n = 40), PSY316 *mpt5* 15.3 (n = 40), and PSY316 *mpt5 SSD1-V* 22.9 (n = 40). (B) Life spans were determined for W303R (◆), W303R *pADH_MPT5* (■), W303R *sir2* (▲), W303R *sir2 pADH_MPT5* (X), W303R *SIR2/URA3* (*), and W303R *SIR2/URA3 pADH_MPT5* (●). This experiment was performed in the W303R strain background because overexpression of *MPT5* from the *pADH_MPT5* plasmid causes slow growth and decreased viability in strain PSY316. Mean life spans and number of cells analyzed were W303R 20.9 (n = 41), W303R *pADH_MPT5* 25.8 (n = 70), W303R *sir2* 11.2 (n = 41), W303R *sir2 pADH_MPT5* 10.1 (n = 70), W303R *SIR2/URA3* 27.4 (n = 40), and W303R *SIR2/URA3 pADH_MPT5* 25.8 (n = 40).

TABLE 3

Effects of *MPT5*, *SIR2*, and *SSD1-V* on silencing

Genotype	Telomere silencing	rDNA silencing
*mpt5*Δ	↑	↓
*sir2*Δ	↓	↓
pADH_MPT5	↓	↑
*pADH_MPT5 sir2*Δ	↓	↓
*ssd1*Δ	↔	↔
SSD1-V	↔	↔

Stated effects on silencing are relative to silencing in the wild-type *ssd1-d* background. Telomere and rDNA silencing were measured by color formation using an *ADE2* marker integrated into subtelomeric or ribosomal DNA. To detect decreased rDNA silencing relative to wild type in the *sir2*Δ strain, an *rpd3*Δ mutation was introduced into the wild-type background.

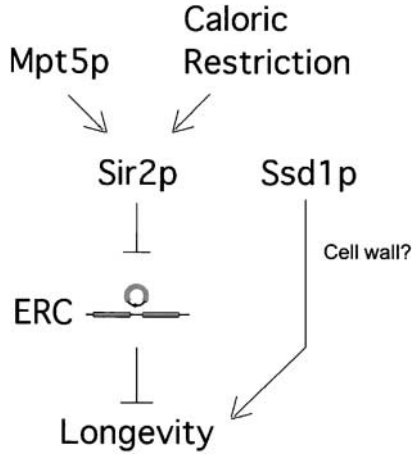

FIGURE 6.—Genetic model for Ssd1p as a regulator of longevity. Ssd1p acts parallel to Sir2p to extend life span. This could involve increasing the cell's resistance to ERCs or could represent an ERC-independent longevity-promoting function. One likely possibility is that *SSD1-V* results in an altered cell-wall structure that allows mother cells to achieve extreme old age.

regulate yeast life span act by altering Sir2p activity or dosage (KAEBERLEIN *et al.* 1999, 2002; LIN *et al.* 2000, 2002). Here we present evidence that *SSD1-V* defines a novel Sir2p-independent pathway necessary for cells to achieve extreme longevity.

Two pathways promoting longevity: We initially began studying *SSD1-V* on the basis of its ability to suppress the temperature sensitivity caused by mutation of the *UTH4/MPT5* gene. Like Sir2p, Mpt5p is limiting for life span in wild-type cells (KENNEDY *et al.* 1997). Overexpression of Mpt5p increases life span and rDNA silencing in a Sir2p-dependent manner (Figure 5B, Table 3), suggesting that Mpt5p promotes longevity by increasing Sir2p activity at the rDNA.

In contrast to overexpression of *MPT5*, addition of a single copy of *SSD1-V* extends life span in both *SIR2* wild-type cells and cells lacking Sir2p (Figure 2, A and C). However, the *sir2 fob1 SSD1-V* strain has a life span that is shorter than that of the *SIR2 FOB1 SSD1-V* strain, demonstrating that Sir2p is required for maximum longevity in *SSD1-V* cells. This is consistent with the observation that *MPT5 SSD1-V* cells have a longer life span than *mpt5 SSD1-V* cells (Figure 5A) and suggests a model whereby Mpt5p and Sir2p function in one pathway to increase life span while Ssd1p functions in a parallel pathway (Figure 6).

Mechanism of life-span extension by *SSD1-V*: How does *SSD1-V* act to extend life span? The effect of Sir2p on life span is, at least partially, due to its ability to deacetylate rDNA histones and inhibit ERC formation (KAEBERLEIN *et al.* 1999). There is no evidence to suggest that *SSD1-V* affects the rate of ERC formation or accumulation. Addition of *SSD1-V* had no detectable effect on rDNA recombination (Figure 3A) or on rDNA silencing (Table 3) in PSY316. Moreover, we failed to detect a decrease in ERC levels in *SSD1-V* cells relative to those in *ssd1-d* cells (Figure 3B). While it is still possible that *SSD1-V* affects ERC replication or segregation specifically in aged cells, we feel that this is unlikely to be the case. An alternative possibility is that *SSD1-V* makes cells more resistant to ERCs, rather than reducing ERC levels. In support of this hypothesis, we often observed that steady-state ERC levels were increased in unsorted *SSD1-V* cells relative to those in wild-type *ssd1-d* cells (Figure 3B), although this was not always the case. The mechanism by which ERCs induce senescence is currently unknown. One hypothesis is that ERCs bind to and titrate key cellular replication or transcription factors away from their normal targets. Alternatively, the rapid amplification of rDNA sequence could alter rRNA transcription and/or processing, resulting in ribosome dysregulation. Ssd1p has been shown to bind RNA and is predicted to have RNase activity (UESONO *et al.* 1997). Perhaps *SSD1-V* alters rRNA or ribosome biogenesis in a manner that makes cells more resistant to ERCs.

One attractive hypothesis is that *SSD1-V* promotes longevity by increasing cell-wall stability and cell integrity. *SSD1-V* suppresses several temperature-sensitive mutations that weaken the cell wall (Table 4) and has been found to directly affect cell-wall composition (WHEELER *et al.* 2003). *SSD1-V* also improves resistance to the cell-wall-perturbing agents CFW and SDS (KAEBERLEIN and GUARENTE 2002), increases the maximum temperature at which PSY316 is capable of growth (Figure 1C), and alters the transcription of cell-wall biosynthetic and structural genes. Perhaps the cell wall becomes limiting in very old cells and *SSD1-V* extends life span by stabilizing it. How might cell-wall stability limit replicative life span? The terminal phenotype of yeast cells in the life-span assay is cell cycle arrest often accompanied by cell lysis (MCVEY *et al.* 2001). Enhanced cell-wall stability

TABLE 4

Reported genetic interactions with *SSD1-V*

Gene	Function	Genetic interaction	Reference
ARL1	Membrane trafficking	ts	Rosenwald *et al.* (2002)
BCK1	Protein kinase involved in cell-wall integrity	ts, other	Costigan *et al.* (1992)
BEM2	GTPase-activating protein required for polarized cell growth	ts	Kim *et al.* (1994)
BUL1	Protein that binds ubiquitin ligase	ts	Yashiroda *et al.* (1996)
CBK1	Protein kinase involved in cell morphogenesis		Du and Novick (2002)
CBC2	Component of cap-binding complex	sl(*sto1*)	Fortes *et al.* (1999)
CCR4	Transcriptional regulator of glucose-repressed and cell-wall genes	sl(*mpt5*), ts	Kaeberlein and Guarente (2002)
CDC28	Cyclin-dependent protein kinase	ts	Sutton *et al.* (1991)
CET1	mRNA capping enzyme	ts	Vincent *et al.* (2003)
CLN1, CLN2	G1 cyclins	G	Cvrckova and Nasmyth (1993)
CYR1	Adenylate cyclase	ts	M. Kaeberlein and L. Guarente, unpublished results
DHH1	ATP-dependent RNA helicase	sl(*elm1*)	Moriya and Isono (1999)
ELM1	Serine/threonine protein kinase that regulates pseudohyphal growth	sl(*dhh1*)	Moriya and Isono (1999)
HDF1	Component of DNA end-joining repair pathway	ts	M. Kaeberlein and L. Guarente, unpublished results
JNM1	Protein required for proper nuclear migration during mitosis	ts	Wilson *et al.* (1991)
LAS1	Essential gene required for bud formation and morphogenesis	ts	Doseff and Arndt (1995)
LUV1	Protein involved in protein sorting in the late Golgi	sl(*rbl2*)	Smith *et al.* (1998)
MEP1, MEP2	Ammonium permeases involved in regulating pseudohyphal growth	G	Lorenz and Heitman (1998)
MPT5	Protein required for high temperature growth and normal life span	ts, sl(*ccr4, swi4, swi6*)	Kaeberlein and Guarente (2002)
PAG1	Protein that functions with Cbk1p to regulate cell morphogenesis	D	Du and Novick (2002)
PDE2	3′,5′-cyclic-nucleotide phosphodiesterase	Miscellaneous	Wilson *et al.* (1991)
PPH21, PPH22	Catalytic subunits of protein phosphatase 2A	ts, other	Evans and Stark (1997)
PRP18	U5 snRNA-associated protein	ts	Luukkonen and Seraphin (1999)
PRP22	Pre-mRNA splicing factor	ts	Vincent *et al.* (2003)
RBL2	Putative tubulin cofactor A	sl(*luv1*)	Smith *et al.* (1998)
RDS3	Spliceosome component	ts	Vincent *et al.* (2003)
RLM1	Transcription factor downstream of MPK1	ts	Watanabe *et al.* (1995)
RPC31	RNA polymerase III	ts	Stettler *et al.* (1993)
RPC53	RNA polymerase III		Stettler *et al.* (1993)
RPD3	Histone deacetylase component of the Rpd3p-Sin3p complex	sl(*swi6*)	Vannier *et al.* (2001)
RPT4	Proteosome ATPase	ts	McDonald *et al.* (2002)
RRD1	Phosphotyrosyl phosphatase activator	sl(*rrd2*)	Rempola *et al.* (2000)
RRD2	Protein involved in rapamycin sensitivity	sl(*rrd1*)	Rempola *et al.* (2000)

(*continued*)

may prevent cell lysis late in life and allow additional cell divisions to occur.

Genetic diversity and the study of aging: The data presented here identify a genetic polymorphism that has a profound effect on mother-cell life span. Genetic polymorphisms have also been proposed to affect the likelihood of achieving extreme longevity in human populations (*e.g.*, Puca *et al.* 2001), as well as in other model systems. Since both *ssd1-d* and *SSD1-V* allele types have been isolated from natural yeast populations, the *SSD1* locus represents a true polymorphic locus affecting longevity. In the past, researchers studying aging in yeast have tended to avoid using long-lived wild-type backgrounds. We speculate that the majority (if not all) of these shorter-lived yeast strains carry *ssd1-d* alleles. A comprehensive reevaluation of previously identified

TABLE 4

(Continued)

Gene	Function	Genetic interaction	Reference
SDS3	Component of the Rpd3p-Sin3p histone deacetylase complex	sl(*swi6*)	Vannier *et al.* (2001)
SIN3	Component of the Rpd3p-Sin3p histone deacetylase complex	sl(*swi6*)	Vannier *et al.* (2001)
SIT4	Phosphatase required for G1-S transition	L	Sutton *et al.* (1991)
SLC5	Unknown	ts	Vincent *et al.* (2003)
SLG1	Protein required for maintenance of cell-wall integrity	ts	Jacoby *et al.* (1998)
SLT2	MAP kinase that functions downstream of BCK1/SLK1	ts	Martin *et al.* (1996)
SLY1	Protein involved in vesicle trafficking	ts	Kosodo *et al.* (2001)
SMC2	Subunit of condensin protein complex	ts	Strunnikov *et al.* (2001)
SMC4	Subunit of condensin protein complex	ts	Strunnikov *et al.* (2001)
SNP1	U1 snRNA-associated protein	ts	Luukkonen and Seraphin (1999)
STO1	Component of cap-binding complex	sl(*cbc2*)	Fortes *et al.* (1999)
SWI4	Cell-cycle-specific transcription factor	ts, sl(*mpt5*)	Kaeberlein and Guarente (2002)
SWI6	Cell-cycle-specific transcription factor	ts, sl(*mpt5*)	Kaeberlein and Guarente (2002)
U5AI	U5 snRNA	ts	Luukkonen and Seraphin (1999)
YPT1	GTP-binding protein involved in the secretory pathway	ts	Li and Warner (1998)
YPT6	GTP-binding protein involved in the secretory pathway	ts	Li and Warner (1998)

Reported effects of addition of *SSD1-V* are shown. ts, suppresses temperature sensitivity; sl (*x*), suppresses synthetic lethality between given gene and gene *x*; G, improves growth; L, suppresses lethality; D, causes death.

mutations affecting life span in a long-lived *SSD1-V* background would be of value to the field.

SSD1-V confers extreme longevity on yeast mother cells by a pathway independent of Sir2p. Sir2 proteins have been found to extend life span in animals and, like *SIR2*, *SSD1* homologs are present in yeast, worms, flies, and mammals. Might *SSD1* family members also promote longevity outside of yeast? The mechanism by which *SSD1-V* cells achieve up to 85% longer life span is still unknown. Further work should be devoted to testing candidate longevity genes regulated by *SSD1-V* and to defining the molecular function of Ssd1p in cells.

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